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Optical monitoring of therapeutic drugs with a novel fluorescence-based POCT device

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Abstract

A novel optical biochip for immunosuppressants detection in transplanted patients is described. The optical working of the chip, based on total internal reflection fluorescence and consisting of two bonded polymeric parts, a Zeonex 330 slide and a Topas foil was demonstrated. In parallel, a heterogeneous competitive immunoassay for tacrolimus was implemented using a thirteen-microchannel optical chip interrogated by an in-house optical platform based on fluorescence anisotropy and the bioassay conditions were optimized: a limit of detection of 0.11 ng/mL, a limit of quantification of 0.57 ng/mL and a coefficient of variation of 25% were achieved.

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Keywords: immunosuppressants; tacrolimus; optical biochip; fluorescence anisotropy

1. Introduction

The measurement of immunosuppressants is essential in transplanted patient in order to avoid the rejection of the transplanted organ [1]. The correct administration window is very narrow and changes from patient to patient: if a low dosage is ineffective, a high dosage can cause an excessive inhibition of the immune response, leading to an increased risk for the patient life. Generally a single drug is not sufficient and a combination of immunosuppressive drugs is necessary, each of them with a different target [2]. Typical examples are given by the inhibitors of

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calcineurin, a protein associated to the activation of the T cells of the immune system, such as Cyclosporin A and Tacrolimus (FK 506), by the inhibitors of the mammalian mTOR proteins, rapamycin (sirolimus) and everolimus and by mycophenolic acid, which inhibits an enzyme needed for the growth of T- and B-cells.

General practice is the periodical administration of the drugs (e.g. twice daily or, in case the drugs are applied as an infusion, over several hours) with the measurement of the drug concentration in whole blood or plasma before its next administration with the purpose to avoid overdosing. This provides the information on the trough level, which is the lowest concentration reached by a drug before the next dose is administered. Recent studies showed that higher clinical indication is given by the area under the concentration time curve (AUC) of immunosuppressant concentrations, since this value is better correlated with efficiency and side effects of immunosuppressive therapy than the trough level. It is apparent that a continuous monitoring of these analytes would provide the best information to physicians in order to define their appropriate dosage, providing the correct information not only on the actual drug levels right value but also on the pharmacokinetics [3]. On the other hand, a continuous measurement of these bioanalytes is impossible, being the measurement based on laboratory immunoassay or high-performance liquid chromatography (HPLC) with ultraviolet (UV) or mass spectrometry (MS), with the last one considered at the moment the reference method. Also the possibility of the measurements of these bioanalytes close to the bed patients, without the delivery of the biological samples to a central lab, would imply an evident step forward in this area since it would provide the result of the measurement in a shorter time. From this point of view, immunoassay is a very promising technique, being many immunoassay-based POCT devices already available on the market.

With the final purpose of the development of a POCT device for the detection of immunosuppressive drugs, a novel optical biochip was developed, based on total internal reflection fluorescence (TIRF) and at the same time optimized conditions for tacrolimus detection were achieved by the implementation of a heterogeneous competitive immunoassay using a thirteen-microchannel optical chip interrogated by an optical platform based on fluorescence anisotropy.

2. Materials and methods

All the used chemicals were of analytical-reagent grade and were purchased from Sigma, if not otherwise specified. Prionex® Calbiochem was from Merck Millipore (Darmstadt, Germany). Derivative of tacrolimus (tacrolimus-COOH) was provided by the group of Prof. G. Orellana (Universidad Complutense de Madrid, Spain) [4]. Tacrolimus and anti-tacrolimus monoclonal antibody from mouse (IgM, clone FK1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Donkey anti-mouse IgM labelled with Alexa Fluor® 647 (Jackson Immunoresearch, West Grove, PA) was used as secondary antibody. Streptavidin labelled with ATTO647N was purchased from Jena Bioscience (Jena, Germany).

A preliminary evaluation for the optimization of different parameters for the tacrolimus assay was carried out on optical biochips in PMMA interrogated with an in-house stand-alone optical platform developed within the European project Careman [5]. The chip, produced by injection molding, is constituted by 13 microchannels through which the sample flows. The sensing layer, where the immunochemical reaction takes place, is located on the upper part of each microchannel which is COOH-functionalized. The tacrolimus derivative was immobilized onto the channels via a carbodiimide crosslinking method: the carboxylic groups on the channels and on the tacrolimus derivative were activated by means of 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (200 mM) and N-Hydroxysuccinimide (NHS) (50 mM) and the immobilization of the activated tacrolimus derivative was conducted via crosslinking with diethylenetriamine 1 M pH 8.5 in H_2O for 1 h. After blocking the surface with Prionex (5 mg/mL), the competitive assay was performed with the following protocol:

- incubation of tacrolimus with anti-tacrolimus Ab (IgM, FK1) (0.001 mg/mL) for 30 minutes;
- pumping of the incubated solution within the chip at a flow-rate of 50 μL/min for 30 minutes;
- pumping of anti-IgM Ab labelled with Alexa Fluor 647 (0.001 mg/mL) at a flow-rate of 50 μL/min for 30 minutes.

Between each step, a washing with PBS and PBS with the addition of Tween 20 (0.05 %) for 1 minute at a flow rate of 200 μL/min was carried out. Regeneration of the channels was conducted with guanidinium hydrochloride 6 M pH 1.5 for 30 sec.

Topas foil (thickness: 140 μm) and Zeonex 330 slide (thickness: 1 mm) were gently provided by microfluidic

ChipShop, Jena, Germany; the prism for optical coupling was an equilateral prism with the dimension of 10 mm (Thorlabs, PS850 - F2 Equilateral Dispersive Prism). Fluorescence detection was performed with an Andor Shamrock 303 Spectrometer; the laser source was an HeNe with emission at 632.8 nm and 10 mW of emitted optical power; the emitted light was filtered by means of a long-pass interference filter (Thorlabs FEL650). The ray tracing analysis was performed by means of an ad hoc Matlab program.

3. Results and discussion

A calibration curve was achieved for tacrolimus, performed onto 5 different PMMA multichannel biochips with the protocol described in Section 2: a limit of detection (LOD) of 0.11 ng/mL, a limit of quantification (LOQ) of 0.57 ng/mL and a coefficient of variation (CV%) of 25% were achieved. The assay was repeated with tacrolimus 10 ng/mL after regeneration and comparable results with respect to the first assay were achieved (Figure 1)

Unspecific adsorption was also evaluated by exposing the sensing surface, coated with the tacrolimus derivative and passivated with Prionex, to the labelled anti-IgM Ab (0.001 mg/ml) without the anti-tacrolimus antibody. The resulting fluorescence (2.3 x 10^6 a.u.), compared with the signal achieved with the anti-tacrolimus antibody (2.4 x 10^7 a.u.), evidences low level of unspecific adsorption. A very low signal (8.1 x 10^4 a.u.) was also observed after exposing the COOH functionalized PMMA surface, solely blocked with the passivating solution, to the labelled anti-IgM Ab (0.001 mg/ml).

Figure 2A shows the design of the novel optical chip based on TIRF: it consists of two parts: a top polymer part properly shaped with a trapezoidal transversal cross section with refractive index n_2 and a bottom thin polymer foil with refractive index n_1. The refractive indices are chosen so that $n_1>n_2$. The microfluidic channel is constituted by a rectangular groove on the bottom side of the trapezium shaped polymer, with the chemical sensing layer located on the lower side of the microchannel. Due to its higher refractive index, the bottom foil is able to guide the excitation light- coming from an external light source - by total internal reflection. In such a way, the guided light excites, by evanescent field, the fluorescent sensing-layer immobilized onto the fluidic microchannel. Thanks to the proper shape of the top part and since the emission of fluorescence is not isotropic, due to the closeness of the fluorophore to the interface [6], the emitted fluorescence is mainly coupled inside the denser plastic foil and is reflected back at the air/plastic interface by total reflection, since the incidence angle is greater than the total reflection angle at this interface. When the reflected light impinges the interface between the plastic foil and the trapezium shaped polymer, since n_1 is greater than n_2, the light is refracted and enters the less dense polymer. With the proper choice of the inclination of the lateral side of the trapezoidal shaped polymer, the light is driven toward the photodetecting apparatus placed on the top of the chip. The multianalyte capability is assured by the presence of multiple channels in parallel.

A two channel prototype was manufactured using a top Zeonex 330 slide (thickness: 1 mm, refractive index: 1.509) and a bottom Topas foil (thickness: 140 μm, refractive index: 1.53), bonded together by means of temperature-based solvent technique. The height, the width and the length of the microchannel are 100 μm, 600 μm, and 10 mm, respectively. The dimensions of the trapezoidal section of the Zeonex 330 are the following: the two

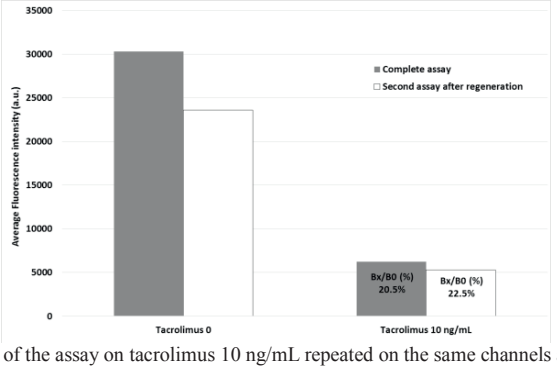

Fig. 1. Comparison among the results of the assay on tacrolimus 10 ng/mL repeated on the same channels after regeneration with guanidinium hydrochloride.

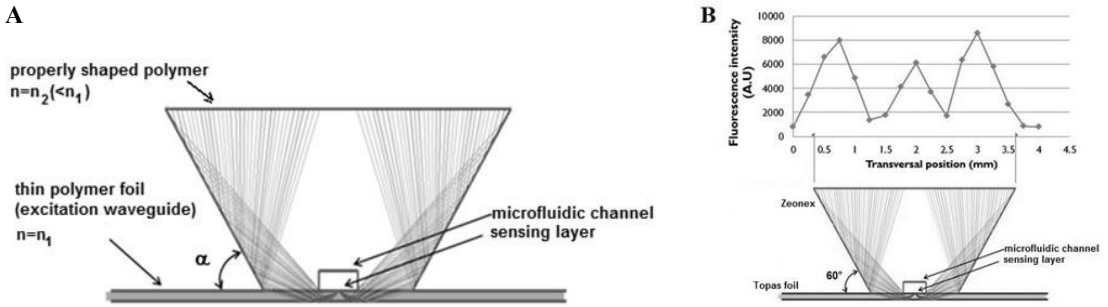

Fig.2. (A) transversal cross section of the channel of the designed optical chip, with the ray tracing simulation of the fluorescence emitted by sensing layer; (B) fluorescence transversal distribution collected at the top of the Zeonex.

basis are 1.47 mm and 3.2 mm and the height is 1.5 mm. The α angle of 60° assures the total reflection of the emitted fluorescent along the lateral sides of the trapezoidal Zeonex. The optical working of the chip was verified by implementing a fluorescent layer constituted by Streptavidin labelled with ATTO647N within the microchannel; prism coupling is used to couple the light from the laser to the Topas film [5]. The fluorescence emitted light was collected from the top of the chip by means of a 200 μm multimode optical fiber, was filtered by means of the long-pass interference filter and detected by the Andor spectrometer.

Figure 2B shows the fluorescence distribution detected on the top of the trapezoidal shaped Zeonex, achieved by means of a transversal scanning with the optical fibre connected to the spectrometer.

4. Conclusions

The design of a novel biochip for immunosuppressants detection in transplanted patients was presented and preliminary tests on the bioassay implementation for tacrolimus detection were successfully performed. The tacrolimus bioassay was characterized by a LOD of 0.11 ng/mL, a LOQ of 0.57 ng/mL and a CV% of 25%.

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